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Mechanism of p21-activated Kinase 6-mediated Inhibition of Androgen Receptor Signaling*

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PAK6 was first identified as an androgen receptor (AR)-interacting protein able to inhibit AR-mediated transcriptional responses. PAK6 is a serine/threonine kinase belonging to the p21-activated kinase (PAK) family implicated in actin reorganization and cell motility, gene transcription, apoptosis, and cell transformation. We investigated the biochemical basis for inhibition of AR signaling by PAK6. We compared the kinase activity of PAK6 with two other well characterized members of the PAK family, PAK1 and PAK4. Like PAK4, PAK6 possesses a constitutive basal kinase activity that, unlike PAK1, is not modulated by the binding of active Rac or Cdc42 GTPases. In order to test the involvement of PAK6 kinase activity in suppression of AR-mediated transcription, we generated kinase-dead (K436A) and kinase-active (S531N) mutants of PAK6. We show that PAK6 kinase activity is required for effective PAK6-induced repression of AR signaling. Suppression does not depend upon GTPase binding to PAK6 and is not mimicked by the closely related PAK1 and PAK4 isoforms. Kinase-dependent inhibition by PAK6 extended to the enhanced AR-mediated transcription seen in the presence of coactivating molecules and to the action of AR coinhibitors. Active PAK6 inhibited nuclear translocation of the stimulated AR, suggesting a possible mechanism for inhibition of AR responsiveness. Finally, we observe that autophosphorylated, active PAK6 protein is differently expressed among prostate cancer cell lines. Modulation of PAK6 activity may be responsible for regulation of AR signaling in various forms of prostate cancer.

Currently, six members of the p21-activated kinase (PAK)[1] family of protein kinases have been identified and can be classified into two groups based on their sequence homology and regulatory properties: group I, including PAKs 1–3, and group II, including PAKs 4–6 (1–3). PAKs are serine/threonine kinases that contain a Cdc42/Rac-interactive binding (CRIB) domain and a Ste20-related kinase domain. The PAK family members have been implicated in the regulation of multiple cellular functions, including actin reorganization, cell motility, gene transcription, cell transformation, apoptotic signaling, and more recently, steroid hormone receptor signaling (see below).

The binding of activated GTP-bound Cdc42 or Rac to group I PAKs dramatically stimulates their ability to phosphorylate exogenous substrates. In contrast, the group II PAKs, PAK4 and PAK5, possess a substantial "basal" kinase activity that is not further stimulated by binding of activated GTPase (4, 5). GTPase binding does mediate kinase relocalization: after binding Cdc42, PAK4 is relocalized to the Golgi (4), and PAK5 shuttles from the microtubule network to actin-rich structures (6). The mechanisms by which PAK6 activity is regulated and the role of PAK6 kinase activity in its biological functions have not yet been studied.

PAK6 was identified by yeast two-hybrid screening as an androgen receptor-interacting protein (7). After androgen stimulation, PAK6 was reported to interact with the ligand binding domain of the androgen receptor (AR) and to translocate to the nucleus along with the AR, where PAK6 inhibits AR-mediated transcription. Northern blot analysis shows that PAK6 is mainly expressed in brain, testis, prostate, and breast tissues (7, 8). PAK6 has also been shown to bind the estrogen receptor (ER) and to inhibit ER-mediated gene transcription (8). Interestingly, the inhibitory effect of PAK6 on AR- and ER-mediated gene transcription is opposite to the transactivation of the ER induced by PAK1-mediated receptor phosphorylation (9).

The AR and ER are hormone-activated transcription factors that belong to the nuclear receptor superfamily (10, 11). The AR has a fundamental role in the development and differentiation of androgen-sensitive tissues and also plays an important role in the pathogenesis of prostate cancer (12). Structurally, the AR is composed of three important functional domains, an N-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a ligand binding domain (LBD). In the absence of androgen, the AR is localized in the cytoplasm in association with heat shock proteins (13, 14). Upon stimulation by dihydrotestosterone (DHT) or DHT analogues, including R1881, heat shock proteins are released, and homodimerization and translocation of the AR to the nucleus occur (15, 16).

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[1] The abbreviations used are: PAK, p21-activated kinase; AR, androgen receptor; ARA, androgen receptor-associated; β-gal, β-galactosidase; CRIB, Cdc42/Rac-interactive binding; DBD, DNA binding domain; DHT, dihydrotestosterone; LBD, ligand binding domain; Luc, luciferase; GST, glutathione *S*-transferase; TAD, transcription activation domain; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; wt, wild type; DTT, dithiothreitol; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; MMTV-LTR, murine mammary tumor virus-long term repeat.

The ligand-bound nuclear AR is capable of mediating transactivation and/or transcriptional repression of target genes. The transcriptional activity of AR is modulated by interaction with cofactors, including coactivators that enhance AR activity and corepressors that inhibit AR activity (17). SRC1, p300, Tip60α, β-catenin, and ARA55 are among these coactivating molecules that have been shown to increase AR-mediated transcription (18–23). The transcriptional activity of the AR can also be regulated either by direct phosphorylation of the receptor and/or by phosphorylation of cofactors (24–28).

In this paper, we investigated the mechanism(s) by which PAK6 inhibits AR-mediated transactivation. By using both kinase-inactive and constitutively kinase-active mutants of PAK6, we showed that inhibition of AR transcriptional activity by PAK6 is dependent on its kinase activity. The binding of Cdc42 GTPase to PAK6 was not required for transcriptional inhibition. The inhibitory effect of PAK6 on AR-mediated transcription was dominant to AR coactivator functions and synergized with corepressors. PAK6 phosphorylated the DNA binding domain of the AR in *in vitro* kinase assays. Finally, we observed that phosphorylated, activated PAK6 protein is expressed differently among the different prostate cancer cell lines. Our results suggest that modulation of PAK6 expression and/or kinase activity may be an important component in the regulation of AR signaling in various forms of prostate cancer.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum, and supplements were from Invitrogen. [γ-^{32}P]ATP (specific activity 4500 mCi/mmol) was from ICN, Costa Mesa, CA. Plasmids for transfection were purified using the Qiafilter purification system of Qiagen, Chatsworth, CA. Thrombin was purchased from Sigma; GDP and GTPγS were from PerkinElmer Life Sciences. For PCR, the Expand High Fidelity PCR system from Roche Applied Science was used.

Cell Cultures—Human cancer cell lines PC-3, PC3MM2 (41), Du145, and HeLa were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μg/ml). MCF-7, ARCaP (42), and LNCaP were maintained in RPMI 1640 with 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μg/ml). PC3gi is a subclone of the human prostate carcinoma cell line PC3.

Plasmid Construction—PAK1, PAK4, and PAK6 were subcloned into pcDNA3-EGFP. PAK6 was inserted into the Myc tag vector pCMV6M. To produce K436A, S531N, and S560E single mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5′ and PAK6-XhoI-3′) and overlapping primer pairs to introduce the desired mutations (for K436A, forward primer 5′-CGC CAG GTG GCC GTC gca ATG ATG GAC GTC AGG-3′; reverse primer 5′-CCT GAG GTC CAT CAT tgc GAC GGC CAC GTG GCG-3′; for S531E, forward primer 5′-GAC ATC AAG AGT GAC aac ATC CTG CTG ACC-3′; reverse primer 5′-GGT CAG CAG GAT gtt GTC ACT CTT GAT GTC-3′; for S560N, forward primer 5′-GTC CCT AAG AGG AAC gag CTG CTG GAA ACC CTC-3′; reverse primer 5′-GGG CGT TCC CAG CAG ctc CTT CCT CTT AGG GAC-3′; and wt PAK6 as a template (lowercase letters indicate the introduced base mutation)). The different fragments were inserted into EcoRI/XhoI-cut pcDNA3-EGFP. To produce the S531N,S560E double mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5′ and PAK6-XhoI-3′), the overlapping primer pairs S560N, and using PAK6 S531N as a template. The fragment was inserted into EcoRI/XhoI-cut pcDNA3-EGFP. The mutations H20L,H23L were introduced by site-directed point mutation using Quickchange kit (Stratagene, La Jolla, CA) according to the manufacturers instruction. Primer sequences are as follows: PAK6(H20L,H23L) 5′-CCA CAC AAC TTC CAG CTC CGT CTC CTC ACC TCC TTC-3′ and its complementary sequence. The reporter plasmid MMTVpA3-Luc, containing the luciferase gene under the control of the steroid hormone-response elements in the MMTV-LTR, was provided by Dr. Richard Pestell (Albert Einstein Medical College, New York). The human AR cDNA, cloned into SV40 promoter-driven expression vector, pSV-hAR, was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). An SV40-driven β-galactosidase reporter plasmid (pSV-β-GAL) (Promega, Madison, WI) was used in this study as an internal control. The mouse SMRTa cDNA, cloned into pCMX, was provided by Dr. Ronald M. Evans (The Howard Hughes Medical Institute, and the Salk Institute for Biological Studies, La Jolla, CA).

Purification of Recombinant Proteins—GST-AR (TAD, DBD, and LBD) and Cdc42 proteins in vector pGEX-2TK were expressed in BL21 cells and purified according to the protocol of Amersham Biosciences. Buffers for the GST-Cdc42 purification contained 1 μM GDP starting from the lysis step, excluding the dialysis buffer. The GST moiety was cleaved off Cdc42 with thrombin at a final concentration of 10 units/ml glutathione beads. Thrombin was removed by incubation with *p*-aminobenzamidine beads (Sigma), and the protein was dialyzed four times against 25 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride. After dialysis, 1 μM GDP was added again, and the purified protein was concentrated by ultrafiltration.

Immunoprecipitation—HeLa cells were seeded on 10-cm cell culture dishes at 50–70% confluency and transfected using LipofectAMINE (Invitrogen). 2 μg of plasmid DNA and 30 μl of LipofectAMINE were used per dish, and the transfection protocol was essentially followed according to the manufacturer's guidelines (Invitrogen). After 30 h the cells were washed with PBS, lysed in 0.5 ml of Lysis Buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and centrifuged for 5 min at 4 °C at 15,000 × *g*, and the supernatant was collected. Protein expression in the lysates was determined by immunoblotting. For precipitations of EGFP, Myc, or FLAG-tagged PAK6 proteins, lysate containing equal amounts of the proteins was incubated with equilibrated protein G beads and anti-EGFP (mAb 3E6; Molecular Probes, Eugene, OR), anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-FLAG (M2; Molecular Probes) antibodies for at least 3 h or overnight at 4 °C. The bead fraction was washed four times with lysis buffer, twice in kinase buffer, and used for kinase assays.

Loading of Cdc42—Cdc42 was loaded with GTPγS or GDP under the following conditions. 5–20 μg of Cdc42 was incubated in 25 mM Hepes/NaOH, pH 7.5, containing 20 mM EDTA and 1 mM GTPγS or GDP for 10 min at 30 °C in a total volume of 25–100 μl. The reaction was stopped by addition of $MgCl_2$ at 100 mM final concentration. GTPγS or GDP-loaded Cdc42 was directly used in kinase or pull-down assay.

In Vitro Kinase Assay—Kinase reactions with immunoprecipitated PAK were performed in kinase buffer (50 mM Hepes/NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT) in a volume of 60 μl with 250 μM [γ-^{32}P]-labeled ATP was used at 10 μCi/reaction. The reactions were incubated for 30 min at 30 °C and stopped by addition of sample buffer. Histones H3/H4 or AR proteins were used as a substrate at 1 μg/reaction.

Western Blot—Cells were lysed and supernatants collected as described above. β-Mercaptoethanol and bromphenol blue were added, and cell lysate proteins were resolved by SDS-PAGE. Proteins were then electroblotted onto nitrocellulose filters, and filters were blocked by incubation for 1 h with 5% bovine serum albumin in Tris-buffered saline, 0.1% Tween 20 and then incubated overnight at 4 °C with anti-EGFP antibody (rabbit antibody, Molecular Probes, Eugene, OR) or anti-PAK6 phospho-antibody (anti-PAK4(Ser-474) anti-PAK5(Ser-602), and anti-PAK6(Ser-560) phospho-antibody 3241; Cell Signaling Technology, Inc. Beverly, MA). Blots were washed three times for 10 min in Tris-buffered saline plus 0.1% Tween 20 and incubated for 1 h in peroxidase-labeled anti-rabbit immunoglobulins. Blots were developed with the use of the enhanced chemiluminescence detection system (Pierce).

Pull-down Assay—Cells were transfected and lysed, and supernatants were collected as described above. Clarified lysate was incubated with either 1 μg of GTPγS or GDP-loaded Cdc42 for 2 h at 4 °C. Anti-EGFP antibody and protein G beads were then added and incubated at 4 °C for another hour. Beads were washed three times in lysis buffer, boiled in SDS sample buffer for 5 min, separated by SDS-PAGE, and subjected to a Western blotting with either an EGFP antibody (rabbit antibody, Molecular Probes) or a Cdc42 antibody (SC-87, Santa Cruz Biotechnology).

AR Transcriptional Activity—The monkey kidney cell line CV-1 containing no detectable levels of endogenous steroid hormone receptor activity was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Hyclone, Denver, CO). Transient transfections were carried out with LipofectAMINE (Invitrogen). Cells were plated on 12-well plates at 50–70% confluency 16 h before transfection, and 400 ng of total plasmid DNA per well was used in the transfection. Approximately 16 h after transfection, the cells were washed and fed with medium containing 5% charcoal-stripped (steroid

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hormone-free) fetal calf serum (Hyclone) in the presence or absence of R1881. Cells were incubated for another 24 h, lysates prepared, and luciferase and galactosidase activities measured with the Luciferase Assay kit (Promega) and Galactolight kit (Tropix, Bedford, MA), respectively.

Immunofluorescence—HeLa cells were plated onto 2-well chamber slides. After 24 h, cells were transiently transfected with wt or mutated EGFP-tagged pcDNA3-PAK6 and pSV-hAR with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. 24 h post-transfection, cells were left untreated or treated with 10 nM R1881 for another 24 h. Cells were fixed for 5 min with 4% paraformaldehyde in PBS and placed in methanol for 1 min, followed by sequential washes with PBS and 1% fetal bovine serum in PBS. Cells were incubated with polyclonal anti-AR antibody (Santa Cruz N20816) for 1 h at room temperature. Cells were washed with PBS and incubated with Texas Red-conjugated anti-rabbit secondary antibody (Molecular Probes). Cells were dried and mounted with fluor mounting medium (Vector Laboratories). Pictures were taken using confocal microscopy.

RESULTS

Analysis of PAK6 Kinase Activity—In order to evaluate the kinase activity of PAK6 and compare it to different members of the PAK family, HeLa cells were transfected with vectors containing wild type (wt) PAK6, PAK1, or PAK4. Equal amounts of EGFP-PAK1wt, EGFP-PAK6wt, and EGFP-PAK4wt were immunoprecipitated with an anti-EGFP antibody, and an *in vitro* kinase assay was performed using histone H3/H4 as an exogenous substrate, in the presence of either GDP-loaded (inactive) or GTPγS-loaded (active) Cdc42. As expected, significant phosphorylation of H3/H4 by PAK1wt was stimulated only in the presence of Cdc42-GTPγS. In contrast, substantial phosphorylation of H3/H4 by PAK6wt and PAK4wt was observed in the presence of both Cdc42-GDP and Cdc42-GTPγS, and the activity was not enhanced by the presence of the active GTPase (Fig. 1*A*). The relative kinase activity of Cdc42-activated PAK1wt was stronger than with PAK6wt or PAK4wt at comparable protein expression levels. An additional slower migrating band was observed with PAK6wt, suggesting that, as with PAK1 and PAK4wt, PAK6wt became autophosphorylated. However, unlike the autophosphorylation of PAK1wt, which is activation (Cdc42-GTPγS)-dependent, autophosphorylation of PAK6wt and PAK4wt was Cdc42-independent.

In order to verify that the presence of the relatively large EGFP tag did not alter the ability of Cdc42 to activate PAK6, HeLa cells were transfected with vectors containing Myc-, FLAG-, or EGFP-tagged versions of PAK6wt, and then protein was immunoprecipitated with the relevant epitope tag antibody. An *in vitro* kinase assay was performed in the presence of Cdc42 loaded with either GDP or GTPγS using histone H3/H4 as an exogenous substrate. As shown in Fig. 1*B*, phosphorylation of H3/H4 mediated by each FLAG-, Myc-, and EGFP-tagged PAK6wt was not modulated by the presence of Cdc42 loaded with either GDP or GTPγS. These results show that PAK6 possesses a substantial basal kinase activity that is not further stimulated by Cdc42 (or Rac; data not shown).

Characterization of PAK6 Mutants—Several PAK6 mutants were generated in an attempt to obtain kinase-inactive and highly kinase-active forms of PAK6. PAK6 (K436A) introduced a lysine-to-alanine mutation at amino acid 436 in the activation loop within the kinase domain (Fig. 2*A*). Mutation of this residue is predicted to inhibit the binding of ATP and abolish kinase activity. PAK6(S560E) converted serine in a predicted autophosphorylation site (based on homology to PAK1) to glutamic acid. PAK6(S531N) introduced a serine-to-asparagine mutation at amino acid 531 in the catalytic loop. Mutation of this residue is predicted to stabilize the catalytic loop, as is the double mutant PAK6(S531N,S560E) (Fig. 2*A*). The EGFP-tagged version of each construct was transiently transfected into HeLa cells; equal amounts of PAK6 protein were immunopurified from cell lysates using EGFP antibody, and an *in vitro* kinase assay was performed using histone H2/H4 as an exogenous substrate. As shown in Fig. 2*B*, PAK6(K436A) exhibited no autophosphorylation nor exogenous kinase activity. Mutation S560E alone did not change the exogenous kinase or autophosphorylation activity of PAK6 from that of PAK6wt. In contrast, the mutation S531N strongly enhanced both exogenous kinase and autophosphorylation activity of PAK6. A similarly enhanced PAK6 activity was observed with the PAK6(S531N,S560E) double mutant. These results suggest that the S531N mutation stabilized the catalytic loop within the kinase domain of PAK6 to increase the kinase activity. The kinase activity of these mutants was not modulated by the presence of Cdc42 loaded with either GDP or GTPγS (Fig. 2*C*).

The ability of an anti-phospho-PAK6 antibody directed against the Ser-560 predicted autophosphorylation site to detect active PAK6 was evaluated. Phosphorylation of the corresponding serine 423 residue in PAK1 was observed after activation by Rac/Cdc42 and reflected PAK1 kinase activity (29–31). HeLa cells were transfected with PAK6wt, K436A, S531N, and S560E, and cell lysates were prepared and analyzed by Western blot using anti-phospho-PAK6 antibody (Fig. 2*D*, *upper panel*). An anti-EGFP antibody was used to confirm similar protein expression levels (Fig. 2*D*, *lower panel*). As expected, PAK6(K436A) kinase-inactive and PAK6(S560E)-modified serine were not detected by the anti-phospho-PAK6 antibody. In

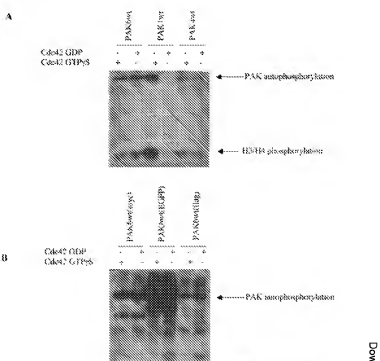

FIG. 1. **Analysis of PAK6 kinase activity.** *A*, HeLa cells were transiently transfected with pcDNA3-EGFP expression vector containing wild type PAK1, PAK4, or PAK6. After cell lysis, the amount of each PAK in the lysate was evaluated by Western blot and densitometry of the common EGFP fusion. Equal amounts of each PAK were incubated with Cdc42 loaded with either GDP or GTPγS, and an *in vitro* kinase assay was performed using histone H3/H4 as substrate. Phosphorylation was detected by autoradiography. *B*, HeLa cells were transfected with pcMV-myc, pcDNA3-EGFP, or pcDNA3-FLAG expression vectors containing PAK6wt. PAK6wt Myc-tagged, EGFP-tagged, or FLAG-tagged proteins were immunoprecipitated with an anti-Myc, anti-EGFP, or anti-FLAG antibody, respectively. Immunoprecipitates were incubated with Cdc42 loaded with GDP or GTPγS, and kinase assays were performed using histone H3/H4 as substrate. Phosphorylations were detected by autoradiography. Results shown are indicative of 3–4 similar experiments.

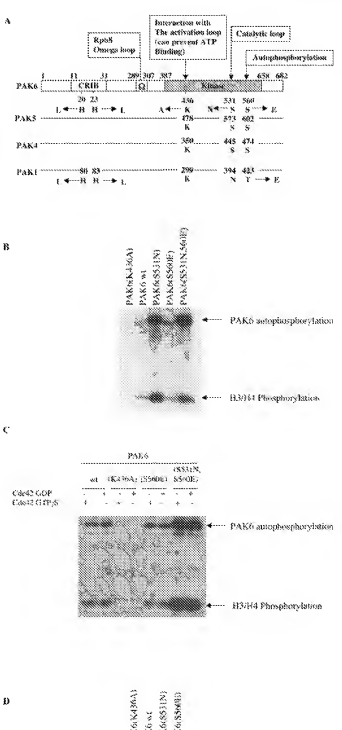

FIG. 2. **Kinase activity of PAK6 mutants.** *A*, schematic representation of PAK6 showing functional domains and point mutations used in this study. An alignment of the kinase domain and the CRIB domain of PAK1, PAK4, PAK5, and PAK6 shows the position of conserved amino acids. Mutation PAK6(K436A) corresponds to the kinase-inactivating mutation of the ATP-binding site K299A in PAK1. Mutation PAK6(S531N) corresponds to the activating mutation in the catalytic loop S445N in PAK4. The mutation S560E in PAK6 corresponds to the activating mutation of the autophosphorylation site T423E in PAK1. The mutations H20L,H23L in PAK6 correspond to mutations H83L,H86L in the CRIB domain of PAK1, which has been shown to abolish the binding of Rac or Cdc42 to PAK1 (3, 33). The Rbp8 Ω loop domain of PAK6, homologous to RNA polymerase subunit 8 Omega loop, is also shown. *B*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A, S531N, S560E, and S531N,S560E. An *in vitro* kinase assay using histone H3/H4 as substrate was performed on the immunoprecipitated PAK6 proteins and then analyzed by autoradiography. *C*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants as indicated. PAK6 proteins were immunoprecipitated and incubated with Cdc42 loaded with GDP or GTPγS, and a kinase assay was performed. Phosphorylations were detected by autoradiography. *D*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A and S531N,S560E. An aliquot of each lysate was analyzed by Western blot using an anti-EGFP antibody (*top panel*) or an antibody recognizing PAK6 protein phosphorylated on serine 560 (*bottom panel*). Results shown in *B–D* are representative of three similar experiments.

contrast, this antibody was able to detect PAK6wt and PAK6(S531N) mutants. These results are consistent with phosphorylation at serine 560 as an indicator reflecting PAK6 kinase activity.

Repression of AR-mediated Transcription by PAK6 Is Dependent on PAK6 Kinase Activity—It has been shown previously (7, 8) that overexpression of PAK6 specifically repressed AR-mediated transcription. To assess whether the kinase activity of PAK6 was required to inhibit AR-mediated transcription, CV1 cells were cotransfected with AR (pSVAR) and PAK6 plasmids, along with a luciferase gene reporter under the control of the steroid hormone-responsive elements in the MMTV-LTR (MMTVpA3-Luc). As shown in Fig. 3*A*, PAK6wt was able to repress in a dose-dependent manner AR transcriptional activity induced by different concentrations of R1881 ranging from 0.1 to 100 nM. wt PAK6 and mutant (S560E), which both have the same relative level of kinase activity, repressed to a similar extent AR transcriptional activity induced by R1881 (Fig. 3*B*). Significantly, PAK6(S531N) and PAK6(S531N,S560E) mutants that exhibited strong kinase activities inhibited ligand-dependent AR transcriptional activity more effectively (*i.e.* by more than 85%) at similar levels of transfected DNA. In marked contrast, kinase-inactive mutant PAK6(K436A) inhibited less than 10–20% of AR-dependent transcriptional activity (Fig. 3*B*). There is thus a correlation between relative kinase activity of PAK6 proteins and their ability to suppress AR-mediated transcription.

We next tested the specificity of the inhibition of AR transcriptional activity by transfecting CV1 cells with wt or dominant active versions of PAK1, PAK4, and PAK6. Fig. 3*C* shows that PAK4 only weakly repressed AR transcriptional activity, whereas PAK1 modestly stimulated AR transcriptional activity. PAK6(S531N) dominant active was used as control. These results provide evidence that the inhibition of AR-mediated transcription is specific to the testis/prostate-enriched PAK6 isoform.

PAK6 Inhibition of AR-mediated Signaling Is Independent of GTPase Binding—The Rho family GTPase Cdc42 has been shown to bind to PAK6, presumably via its CRIB domain (9, 32), but we have demonstrated that this interaction does not affect PAK6 kinase activity. The binding of Cdc42 to PAK4 and PAK5 also does not increase their kinase activity but rather modulates their subcellular localization (4, 6). We wanted to determine whether the interaction of PAK6 with GTPase was important in enabling PAK6 to inhibit AR-mediated transcription. We generated a series of PAK6 constructs in which the CRIB domain was mutated in a conserved pair of histidine residues known to be important for GTPase binding (PAK6(H20L,H23L), PAK6(H20L,H23L,K436A), and PAK6(H20L,H23L,S531N)) (33). The binding of Cdc42 to these PAK6 constructs was analyzed by immunoprecipitation of EGFP-tagged PAK6 mutants in the presence of Cdc42 loaded

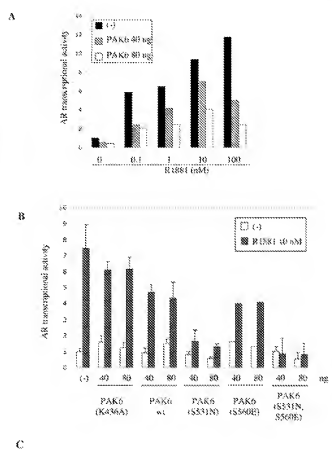

FIG. 3. **Repression of AR-mediated transcription by PAK6 is dependent on PAK6 kinase activity and is specific to PAK6.** *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. The total amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were treated with the indicated concentrations of R1881 (0.1–100 nM) and cell extracts prepared 24 h after stimulation, and luciferase activity was monitored. Luciferase activity is reported as relative light units normalized to β-galactosidase activity. *B*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP expression vector containing PAK6 mutants K436A, wt, S531N, S560E, and S531N,S560E cDNAs. 18 h after transfection cells were treated with 10 nM R1881 (*gray bars*) or left untreated (*white bars*), and luciferase activity was determined (reported as relative light units). *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of expression vector containing wild type (wt) PAK1, -4, -6 or active kinase mutant PAK1(T423E), PAK4(S474E), and PAK6(S531N). Total amount of DNA was normalized with pcDNA3. 18 h after transfection, the cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was determined. The *bar graphs* shown in *B* and *C* represent the mean ± S.E. % inhibition of AR-mediated transcription observed in three separate experiments.

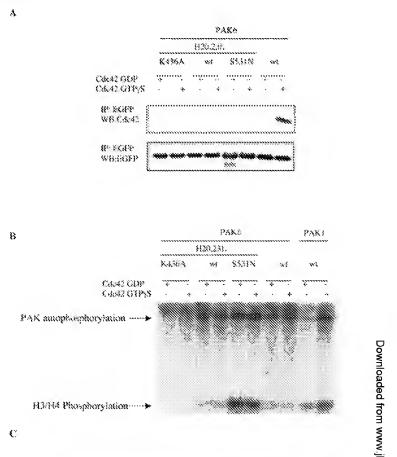

FIG. 4. **PAK6 inhibition of AR-mediated transcription is independent of Cdc42 binding.** *A*, binding of GTPγS loaded Cdc42 to PAK6. HeLa cells were transfected with pcDNA3-EGFP expression vector containing either PAK6wt or PAK6 mutants H20L,H23L,K436A, H20L,H23L, and H20L,H23L,S531N cDNAs. Equal amounts of cell lysate were incubated with Cdc42 loaded with either GDP or GTPγS and immunoprecipitated with an anti-EGFP antibody. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using an anti-Cdc42 antibody or an anti-EGFP antibody. *B*, kinase activity of different PAK6 mutants. HeLa cells were transfected with pcDNA3-EGFP expression vector containing 40 or 80 ng of either PAK6wt, PAK1wt, or PAK6 (H20L,H23L,K436A; H20L,H23L; and H20L,H23L,S531N) cDNAs. PAK proteins were immunoprecipitated and incubated with Cdc42 loaded with either GDP or GTPγS; a kinase assay was performed in the presence of histone H3/H4, and the result was analyzed by autoradiography. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. Total amount of DNA was normalized with pcDNA3. 18 h after transfection cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was monitored. The *bar graph* shown represents the mean ± S.E. % inhibition of AR-mediated transcription by different PAK6 mutants observed in three independent experiments.

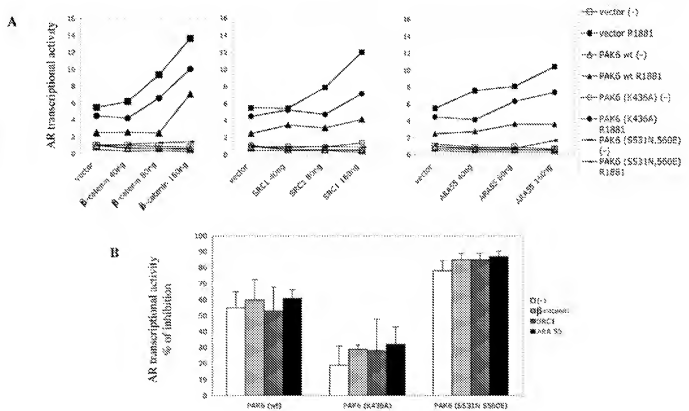

FIG. 5. **PAK6 inhibits AR-mediated transcription in the presence of AR coactivators.** *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pcDNA3-β-catenin, pcDNA3-SRC1, or pSG5-ARA55 constructs (40, 80, and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM of R1881 (*black symbols*) or left untreated (*open symbols*) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of three similar experiments. *B*, the *bar graph* shown represents the mean ± S.E. % inhibition of AR-mediated transcription by PAK6 in presence of AR coactivators. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pCMX-mSMRTa construct (80 and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM R1881 (*black symbols*) or left untreated (*open symbols*) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of four similar experiments.

with either GDP or GTPγS (Fig. 4*A*). PAK6wt was able to pull down Cdc42 loaded with GTPγS, whereas the H20L,H23L mutation abrogated binding of Cdc42 to PAK6. In order to verify that the mutation H20L,H23L did not modify the kinase activity of PAK6, PAK6wt and CRIB mutants were transiently expressed in HeLa cells, and equal amounts of PAK6 were immunopurified from cell lysates using EGFP antibody. An *in vitro* kinase assay using histone H3/H4 was performed in the presence of Cdc42 loaded with either GDP or GTPγS. As shown in Fig. 4*B*, PAK6(H20L,H23L) exhibited the same relative level of exogenous kinase and autophosphorylation activity as did PAK6wt, whereas PAK6(H20L,H23L,K436A) exhibited no kinase activity at all. The mutant H20L,H23L,S531N displayed a strong exogenous kinase and autophosphorylation activity.

We next analyzed the ability of the non-Cdc42-binding H20L,H23L PAK6 mutants to suppress AR-mediated transcriptional activity. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants. Mutation of H20L,H23L did not significantly modify the inhibitory effect of wt and kinase-active (S531N) forms of PAK6 (Fig. 4*C*). These

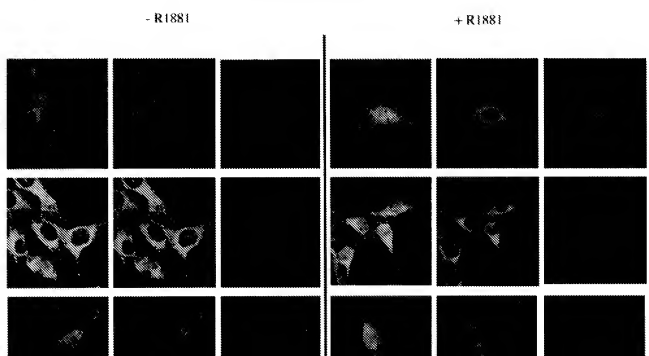

FIG. 6. **PAK6 inhibits AR nuclear translocation.** HeLa cells were transfected with wt or mutated EGFP-PAK6 and AR expression constructs and were cultured in the presence or absence of R1881 (10 nM). AR protein was detected with a polyclonal anti-AR antibody and revealed by Texas Red-conjugated secondary antibody. Pictures of cells were taken with confocal microscopy.

results demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.

PAK6 Inhibits AR-mediated Transcription Enhanced by AR Coactivators and Inhibited with AR Corepressors—AR transcriptional activity can be enhanced by the presence of AR coactivators, including β-catenin, SRC1, ARA55, p300, and Tip60α, which have been shown to bind to distinct regions of the AR and increase AR-mediated transcription. We sought to determine whether PAK6wt and PAK6 mutants were able to inhibit to the same extent AR-mediated transcription in the presence of these differentially binding AR coactivators. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants and serial concentrations of either β-catenin, SRC1, or ARA55. The coactivators β-catenin, SRC1, and ARA55 all increased AR-mediated transcription in a dose-dependent manner (Fig. 5*A*). At a concentration of 160 ng, β-catenin, SRC1, and ARA increased AR-mediated transcription by 2.3-, 2-, and 1.6-fold, respectively. In the presence of the highly active PAK6(S531N,S560E) mutant, these coactivators were not able to increase significantly the transcriptional activity of AR. In contrast, in the presence of PAK6wt or K436A, these coactivators all increased AR transcriptional activity. As shown in Fig. 5*B*, the inhibitory effect of PAK6wt on AR-mediated transcription was 55% in the absence of the coactivator and 60, 53, and 61% in the presence of β-catenin, SRC1, or ARA55, respectively. The inhibitory effect of PAK6(K436A) on AR-mediated transcription was 20% in the absence of the coactivator and 29, 28, and 32% in the presence of β-catenin, SRC1, or ARA55, respectively. The inhibitory effect of the dominant active mutant PAK6(S531N,S560E) on AR-mediated transcription was 80% in the absence of the coactivator and 85, 85, and 87% in the presence of β-catenin, SRC1, or ARA55, respectively. PAK6 also inhibited AR-mediated transcription in the presence of the coactivators p300 and Tip60α (data not shown). In addition, as shown in Fig. 5*C*, PAK6 increased the inhibition of AR signaling seen with the AR corepressor SMRT. These results show that the inhibitory effect of PAK6 on AR-mediated transcription is dominant over the presence of the AR activating cofactors β-catenin, SRC1, ARA55, p300, and Tip60α and are observed in the presence of a corepressor, SMRT, suggesting that PAK6 may act to directly modulate AR function.

Nuclear Translocation of the AR in Response to Androgen Is Inhibited by Kinase-active Mutants of PAK6—To assess the biological function of PAK6 kinase activity, we examined the subcellular localization of the AR after stimulation with androgen in the presence of PAK6 and PAK6 mutants (K436A and S531N) by immunofluorescence. As shown in Fig. 6, in the absence of R1881 the AR localized to the cytoplasm and the nucleus. In control (GFP-transfected) cells, R1881 induced a total nuclear accumulation of the AR, as reported previously (not shown). Similarly, in the presence of the kinase-inactive PAK6(K436A), treatment with R1881 resulted in the total accumulation of the AR in the nucleus. In contrast, upon coexpression of the AR with PAK6wt or highly active PAK6(K531N), R1881-induced translocation of the AR was partially inhibited, with a good deal of receptor remaining in the cytoplasm. These results suggest that PAK6 kinase activity negatively modulates steroid-induced AR translocation into the nucleus.

PAK6 Phosphorylates the AR in the DBD Domain—In order to further investigate the biochemical basis of PAK6-induced inhibition of AR-mediated signaling, we sought to determine whether PAK6 could phosphorylate the AR. To that end, we assessed the ability of PAK6 to phosphorylate a series of recombinant AR proteins (Fig. 7*A*). Highly active PAK6(S531N) was immunoprecipitated from HeLa cell lysates and incubated

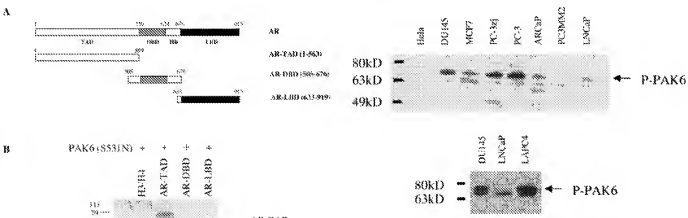

FIG. 7. **PAK6 phosphorylates the AR-DBD domain.** *A*, schematic representation of AR proteins. AR-TAD, AR-DBD, and AR-LBD were expressed in *Escherichia coli* and used as substrates in kinase assays. TAD, DBD, LBD, and hinge region (*HR*). *B*, immunoprecipitated PAK6(S531N) was used in a kinase assay with 1 μg of H3/H4 or 1 μg of the different domains of the AR. Phosphorylation of H3/H4 and the AR fragments was detected by autoradiography. *C*, PAK6wt or PAK6(S531N) was immunoprecipitated from HeLa cells and incubated with 1 μg of H3/H4 or DBD-AR purified from *E. coli*, and a kinase assay was performed. Phosphorylation of H3/H4 and the DBD fragments were detected by autoradiography.

with recombinant AR proteins in an *in vitro* kinase assay. PAK6(S531N) strongly phosphorylated a construct containing the DBD of the AR (Fig. 7*B*). In contrast, no phosphorylation of the AR TAD or the AR LBD could be detected. PAK6wt also phosphorylated the DBD-containing fragment, although phosphorylation was weaker than with PAK6(S531N) (Fig. 7*C*). These results suggest that PAK6 could modify AR activity through the direct phosphorylation of the DNA-binding element, thereby potentially preventing transcriptional regulation from taking place.

PAK6 Protein Expression in Prostate Cancer Cell Lines—To better understand the potential biological role(s) of PAK6, we analyzed the expression of PAK6 protein in different cancer cell lines. The antiphospho-PAK6 (Ser(P)-560) antibody was used to analyze PAK6 in prostate cancer cell lines DU145, PC-3zj, PC-3, ARCaP, PC3MM2, LNCaP, and LAPC4, breast cancer cell line MCF7, and the uterine cancer cell line HeLa (Fig. 8). As shown in Fig. 2*D*, this antibody was able to detect active versions (PAK6wt and PAK6(S531N)) but did not recognize the inactive PAK6(K436A) mutant. Phospho-PAK6 was detected most abundantly in the LAPC4 cell line. High expression/activity levels were also detected in the DU145, PC-3, and PC-3zj lines. MCF7 and ARCaP expressed only a small amount of phospho-PAK6, and no expression was detected in HeLa, LNCaP, and PC3-mm2 cells. These results indicate that the expression and/or activity level of PAK6 in prostate cell lines is highly variable and suggest that PAK6 could be involved in differential regulation of AR signaling in these cells.

FIG. 8. **PAK6 protein expression in human cancer cell lines.** A Western blot was performed on equal protein amounts of lysates from prostate cancer cell lines (DU145, PC3zj, PC3, Arcap, PC3MM2, Lncap, and LapC4), breast cancer cell lines (MCF7), and uterus cancer cells line (HeLa) by using antibody recognizing PAK6 protein phosphorylated on serine 560.

DISCUSSION

In this study, we have investigated the mechanism by which the protein kinase PAK6 inhibits AR-mediated transcription. In order to better characterize the regulation of this serine/threonine kinase, we compared the kinase activity of PAK6 to PAK1 and PAK4, two well characterized members of the group I and group II PAK protein family, respectively. It is well established that the binding of activated Rac and Cdc42 GTPases to PAK1 markedly stimulates its kinase activity, both autophosphorylation and activity toward exogenously supplied substrates (3, 33, 34). In contrast, Rac or Cdc42 GTPase binding to PAK4 does not stimulate the already rather high basal kinase activity (4). We found that, like PAK4, PAK6 exhibits a constitutive kinase activity that is not increased by active Cdc42 (or Rac, not shown) GTPase (Figs. 1*A* and 2*D*). These findings are in accordance to BLAST comparisons showing that, like PAK4 and PAK5, PAK6 lacks the autoinhibitory domain adjacent to the CRIB domain in the group I PAKs whose release by GTPase binding accounts for GTPase-dependent kinase activation.

Whereas two prior studies (7, 8) have described the ability of PAK6 to suppress transcriptional signaling downstream of the androgen and estrogen nuclear receptors, the mechanism(s) underlying this effect have not been determined. We showed that mutation of PAK6 (PAK6(S531N)) to increase its kinase activity relative to the wild type protein dramatically increased its inhibitory effect on AR-mediated transcription (Fig. 3). Conversely, abrogating PAK6 kinase activity (PAK6(K436A)) considerably decreased its inhibitory effect on AR transcriptional activity. These results demonstrate that PAK6 kinase activity is involved in inhibition of AR-mediated transcription. It is also interesting to notice that the kinase-dead mutant of PAK6 (K436A) is still able to inhibit ~20% of AR transcriptional activity (Figs. 3 and 4). This result suggests that PAK6 is also able to suppress the transcriptional activity of the AR by a phosphorylation-independent mechanism. Neither PAK1 nor PAK4 was effective at inhibiting AR signaling, suggesting a mechanism specific to PAK6 is operative. Both prior studies

have indicated a physical interaction of PAK6 with the AR, including a region between the hinge and LBD. By BLAST comparison we have found that PAK6 possesses a unique region localized between amino acids 289 and 307. This domain is homologous to the Ω loop of Rbp8, one of the subunits of RNA polymerase (35, 36). We speculate that this domain could also be important for the PAK6 and AR interaction.

Because the regulation of transcription by the hormone-bound AR requires relocalization of the receptor to the nucleus, we considered the possibility that the binding of GTPase to PAK6 might play some role in this translocation process. We mutated conserved residues within the PAK6 CRIB domain known to be critical for GTPase binding (H20L,H23L). We established that this mutation impeded the binding of Cdc42 to PAK6 without affecting the inhibitory action of PAK6 on AR transcriptional activity (Fig. 4). These results demonstrate that Cdc42 binding is not required for PAK6-mediated inhibition of AR transcriptional activity, and also suggest that PAK6 activity is not modulated by GTPase-dependent relocalization within the cell. It is possible that the interaction of PAK6 with Cdc42 modulates an as yet unknown aspect of PAK6 function that is independent of the observed regulation of AR transcriptional activity.

The inhibition of AR signaling by PAK6 might be the result of a direct effect on the AR or an indirect effect on other AR-associated proteins. To address this issue, we studied the effect of PAK6 on AR signaling in the presence of different AR-associated coactivator and corepressor molecules, β-catenin, SRC1, ARA55, p300, Tip60α, and SMRT, all of which have been reported to increase or decrease AR transcriptional activity (Fig. 5). The presence of these coregulators did not modify the relative inhibitory effect of PAK6 and PAK6 mutants (K436A and S531N) on AR transcriptional activity. These results indicate that the mechanism for AR inhibition by PAK6 does not involve an indirect effect on the AR coactivators, β-catenin, SRC1, and ARA55, and that inhibition occurs through a common component utilized by each coactivator or corepressor (*i.e.* the receptor itself).

Given the fact that PAK6 inhibition of AR-mediated transcription is dependent on its kinase activity and is independent of AR coactivators, we examined the effects of PAK6 on AR nuclear translocation (Fig. 6). Kinase-active versions of PAK6 partially blocked steroid-induced nuclear translocation of the receptor. The lack of nuclear AR would tend to inhibit the transcriptional activity of the receptor. Interestingly, we did not observe steroid-induced nuclear translocation of PAK6, as had been originally reported by Yang *et al.* (7). We cannot rule out that this difference is because of cell type differences or to the use of GFP-tagged PAK6 in the current studies.

We also tested whether PAK6 was able to directly phosphorylate the AR (Fig. 7). We found that wild type and the highly active PAK6 mutant (S531N) were able to phosphorylate a fragment of the AR including the DBD and the hinge domains *in vitro*. It has been reported that PAK6 binds to a region between the hinge region and LBD of the AR. This suggests a scenario in which the *in vivo* binding of PAK6 to this region positions PAK6 to phosphorylate the AR and regulate activity of the nearby DBD domain. Because this region is important for DNA binding by the AR, phosphorylation in this domain by PAK6 could inhibit the binding of AR to DNA and, by this mechanism, suppress its transcriptional activity. There are several potential phosphorylation sites for PAK6 in the AR DBD domain. Of particular interest, Gioeli *et al.* (37) have reported that Ser-650 in the DBD is phosphorylated in response to epidermal growth factor receptor stimulation, which is known to recruit PAK (38). A Ser-650 → Ala mutation has been suggested previously by Zhou *et al.* (39) to regulate AR transactivation of the mouse mammary tumor virus promoter when suboptimal levels of steroid were used. This phosphorylation site in the hinge region is conserved in many of the steroid receptors (40). The identification of the relevant PAK6 phosphorylation sites in the AR and the investigation of this potential regulatory mechanism will be a subject of future studies.

We have shown by using PAK6 mutant proteins of varying activity that serine 560 phosphorylation reflects the kinase activity of PAK6. We used a phosphoserine 560 PAK6 antibody to examine in prostate cancer cell line the expression of active PAK6 (Fig. 7). We found that expression of phosphorylated PAK6 in these cell lines is quite variable. Interestingly, we did not detect phospho-PAK6 expression in the androgen-sensitive cell line LNCaP, but we observed a strong expression of phospho-PAK6 in the androgen-insensitive cell lines DU145 and PC3. These results suggest that PAK6 up-regulation might account for the development and/or maintenance of androgen independence, which is known to be associated with more aggressive tumors. In this regard, the activity common to the PAK family members of modulating cell motility and dynamics may come into play for PAK6 as well. It will be of interest in future studies using a larger sampling of prostate cancer cell lines and tumor tissues to assess the relationship of PAK6 activity to AR phosphorylation state, responsiveness to androgens, and the growth rates and metastatic capabilities of these cancer cells.

In conclusion, this study demonstrates that PAK6 inhibition of AR-mediated transcription is dependent on the kinase activity of PAK6 and is specific to this PAK isoform. PAK6 may act by multiple mechanisms to antagonize transcriptional activity of the AR. Most importantly, we present evidence that expression of active PAK6 in cancer prostate cells is variable but may correlate with androgen sensitivity of the lines. These data suggest that modulation of PAK6 activity may be an important mechanism contributing to the regulation of AR signaling in various forms of prostate cancer.

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REFERENCES

1. Jaffer, Z. M., and Chernoff, J. (2002) *Int. J. Biochem. Cell Biol.* **34,** 713–717
2. Bagrodia, S., and Cerione, R. A. (1999) *Trends Cell Biol.* **9,** 350–355
3. Knaus, U. G., and Bokoch, G. M. (1998) *Int. J. Biochem. Cell Biol.* **30,** 857–862
4. Abo, A., Qu, J., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998) *EMBO J.* **17,** 6527–6540
5. Pandey, A., Dan, I., Kristiansen, T. Z., Watanabe, N. M., Voldby, J., Kajikawa, E., Khosravi-Far, R., Blagoev, B., and Mann, M. (2002) *Oncogene* **21,** 3939–3948
6. Cau, J., Faure, S., Comps, M., Delsert, C., and Morin, N. (2001) *J. Cell Biol.* **155,** 1029–1042
7. Yang, F., Li, X., Sharma, M., Zarnegar, M., Lim, B., and Sun, Z. (2001) *J. Biol. Chem.* **276,** 15345–15353
8. Lee, S. R., Ramos, S. M., Ko, A., Masiello, D., Swanson, K. D., Lu, M. L., and Balk, S. P. (2002) *Mol. Endocrinol.* **16,** 85–99
9. Wang, R. A., Mazumdar, A., Vadlamudi, R. K., and Kumar, R. (2002) *EMBO J.* **21,** 5437–5447
10. Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
11. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol.* **10,** 1167–1177
12. Quigley, C. A., De Bellis, A., Marschke, K. B., el-Awady, M. K., Wilson, E. M., and French, F. S. (1995) *Endocr. Rev.* **16,** 271–321
13. Sanchez, E. R. (1990) *J. Biol. Chem.* **265,** 22067–22070
14. Ohara-Nemoto, Y., Nemoto, T., Sato, N., and Ota, M. (1988) *J. Steroid Biochem.* **31,** 295–304
15. Jenster, G., van der Korput, H. A., van Vroonhoven, C., van der Kwast, T. H., Trapman, J., and Brinkmann, A. O. (1991) *Mol. Endocrinol.* **5,** 1396–1404
16. Jenster, G. (1999) *Semin. Oncol.* **26,** 407–421
17. Dotzlaw, H., Moalven, U., Mink, S., Cato, A. C., Iniguez Lluhi, J. A., and Baniahmad, A. (2002) *Mol. Endocrinol.* **16,** 661–673
18. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11540–11545
19. Fu, M., Wang, C., Reutens, A. T., Wang, J., Angeletti, R. H., Siconolfi-Baez, L., Ogryzko, V., Avantaggiati, M. L., and Pestell, R. G. (2000) *J. Biol. Chem.*

275, 20853–20860
20. Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., and Brown, M. (1994) *Science* **264,** 1455–1458
21. Yang, F., Li, X., Sharma, M., Sasaki, C. Y., Longo, D. L., Lim, B., and Sun, Z. (2002) *J. Biol. Chem.* **277,** 11336–11344
22. Fujimoto, N., Yeh, S., Kang, H. Y., Inui, S., Chang, H. C., Mizokami, A., and Chang, C. (1999) *J. Biol. Chem.* **274,** 8316–8321
23. Brady, M. E., Ozanne, D. M., Gaughan, L., Waite, I., Cook, S., Neal, D. E., and Robson, C. N. (1999) *J. Biol. Chem.* **274,** 17599–17604
24. de Ruiter, P. E., Teuwen, R., Trapman, J., Dijkema, R., and Brinkmann, A. O. (1995) *Mol. Cell. Endocrinol.* **110,** R1–R6
25. Nazareth, L. V., and Weigel, N. L. (1996) *J. Biol. Chem.* **271,** 19900–19907
26. Zhou, Z. X., Sar, M., Simental, J. A., Lane, M. V., and Wilson, E. M. (1994) *J. Biol. Chem.* **269,** 13115–13123
27. Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. (1994) *Cancer Res.* **54,** 5474–5478
28. Wang, X., Yang, Y., Guo, X., Simpson, E. R., Hsu, C. L., Tsai, M. Y., Yeh, S., Wu, G., Guo, Y., and Chang, C. (2002) *J. Biol. Chem.* **277,** 15426–15431
29. Yu, J. S., Chen, W. J., Ni, M. H., Chan, W. H., and Yang, S. D. (1998) *Biochem. J.* **334,** 121–131
30. Catti, A., Huang, Z., Tuazon, P. T., and Traugh, J. A. (1999) *J. Biol. Chem.* **274,** 8022–8028
31. Zenke, F. T., King, C. C., Bohl, B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* **274,** 32565–32573
32. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) *Nature* **367,** 40–46
33. Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997) *Curr. Biol.* **7,** 202–210
34. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) *Trends Cell Biol.* **11,** 220–230
35. Briand, J. F., Navarro, F., Rematier, P., Boschiero, C., Labarre, S., Werner, M., Shpakovski, G. V., and Thuriaux, P. (2001) *Mol. Cell. Biol.* **21,** 6056–6065
36. Voutsina, A., Riva, M., Carles, C., and Alexandraki, D. (1999) *Nucleic Acids Res.* **27,** 1047–1055
37. Ciocli, D., Fiearro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, A. D., White, F. M., Christian, R. E., Settlage, R. E., Shabanowitz, J., Hunt, D. F., and Weber, M. J. (2002) *J. Biol. Chem.* **277,** 29304–29314
38. Puto, L. A., Pestonjamasp, K., King, C. C., and Bokoch, G. M. (2003) *J. Biol. Chem.* **278,** 9388–9393
39. Zhou, Z. X., Kemppainen, J. A., and Wilson, E. M. (1995) *Mol. Endocrinol.* **9,** 605–615
40. Denner, L. A., Schrader, W. T., O'Malley, B. W., and Weigel, N. L. (1990) *Cancer Gene Ther.* **8,** 497–505
41. Cao, G., Su, J., Lu, W., Zhang, F., Zhao, G., Marteralli, D., and Dong, Z. (2001) *Cancer Gene Ther.* **8,** 497–505
42. Zhau, H. Y., Chang, S. M., Chen, B. Q., Wang, Y., Zhang, H., Kao, C., Sang, Q. A., Pathak, S. J., and Chung, L. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15152–15157